Effect of Vitamin A Deficiency on Host Intestinal Immune Response to *Eimeria acervulina* in Broiler Chickens[1]

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ABSTRACT The effects of vitamin A (VitA) deficiency on the host intestinal immune response and disease susceptibility to coccidiosis were investigated in broiler chickens following oral infection with *Eimeria acervulina* (*EA*). Day-old male broilers were fed milo-soybean meal diets either with 8,000 IU VitA/kg feed (CONT) or without added VitA (A-DEF). At 25 d, a group of randomly selected birds from each treatment was inoculated orally with *EA*-sporulated oocysts. Intestinal immune response was assessed by the changes in the duodenum intraepithelial lymphocyte (IEL) subpopulations using flow cytometry at 35 d in infected and noninfected birds. Concanavalin A (ConA)-induced spleen lymphocyte proliferation was tested using dimethylthiazol diphenyltetrazolium bromide colorimetric assay. Whether challenged or not with *EA*, A-DEF birds had fewer IEL expressing the surface markers CD3, CD4, CD8, $\alpha\beta$TCR, and $\gamma\beta$TCR. Without *EA* challenge, A-DEF birds had more surface IgA-expressing cells than CONT birds. Upon challenge, A-DEF chickens showed lower $CD4^+$ IEL than CONT chickens. Following *EA* infection, $CD8^+$ IEL increased in the CONT group, whereas no change was found in $CD8^+$ IEL of A-DEF birds. A higher number of *EA* oocysts was recovered from A-DEF birds than from CONT birds (9.2×10^8 vs 5.4×10^8, respectively; $P \leq 0.05$). Serum samples taken 10 d post challenge showed higher antibody level against a recombinant coccidial antigen in A-DEF birds than in CONT birds. The A-DEF birds showed depressed ConA-induced lymphoproliferation response and produced lower serum interferon-γ than CONT birds. These data show that VitA deficiency compromised local immune defenses of challenged birds, as reflected in lymphocyte profiles, oocyst shedding, and interferon-γ levels in A-DEF birds.

(*Key words*: intestinal intraepithelial lymphocytes, vitamin A deficiency, *Eimeria acervulina*, broiler immunity, interferon-γ)

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INTRODUCTION

In recent years, the avian gut-associated lymphoid tissue (GALT) has gained more attention as one of the major immunological systems in chickens (reviewed by Schat and Myers, 1991; Lillehoj and Trout, 1996; Yun and Lillehoj, 2000). Intraepithelial lymphocytes (IEL) are an important component of the GALT due to their close proximity to the gut lumen, which is a major route of entry and a replication site for many economically important pathogens (e.g., coccidia) (Yun and Lillehoj, 2000). Compromising IEL, therefore, would negatively impact general health and increase the susceptibility of chickens to enteric infections.

Many dietary factors, such as vitamin A (VitA), contribute to the integrity of the gut and its associated immune system. Low dietary VitA depressed in vitro T-lymphocyte responses to mitogens (Friedman and Sklan, 1989a; Sklan et al., 1994), specific antibody (Ab) production to protein antigens (Ag) (Friedman and Sklan, 1989b; Sklan et al., 1994), and response to viral vaccines (Davis and Sell, 1989). Erasmus and Scott (1960) reported higher mortality from *Eimeria acervulina* (*EA*) and *E. tenella* infections in chicks fed a low-VitA diet compared to those on a high-VitA diet. Furthermore, CD4:CD8 T-lymphocyte ratios were significantly reduced in chickens fed a low-VitA diet (Lessard et al., 1997).

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Abbreviation Key: A-DEF = vitamin A-deficient; Ab = antibody; Ag = antigen; CMF-HBSS = calcium and magnesium-free Hank's balanced salt solution; ConA = concanavalin A; CONT = control; *EA* = *Eimeria acervulina*; FCS = fetal calf serum; IEL = intraepithelial lymphocytes; IL = interleukin; IFN-γ = interferon-γ; mAb = monoclonal antibody; OD = optical density; RT = room temperature; TCR = T cell receptor; VitA = vitamin A; PBS-T = PBS-Tween-20.

TABLE 1. Composition of the vitamin A-deficient diet

Ingredient	%
Milo[1]	55.6
Soybean meal (48% protein)	37.2
Soybean oil	3.5
DL-Methionine	0.3
Lysine	0.1
Dicalcium phosphate	1.5
Ground limestone	1.0
Choline chloride	0.1
Sodium chloride	0.4
Mineral premix[2]	0.1
Vitamin premix[3]	0.3
Calculated composition	
ME kcal/kg	3,050
CP %	21.5
Crude fat %	5.3
Crude fiber %	2.7

[1]Southern States, Sykesville, MD. Milo analyzed composition: ME = 3,280 kcal/kg; CP = 7.1%; Fat = 2.9%; moisture = 13.3%.

[2]Minerals premix composition: Ca 9.8%; Mn 12%; Fe 4%; Cu 2%; Zn 21%; I 3 ppm; Co 50 ppm.

[3] 3 Vitamins (amount per kg diet when used at 0.3%): cholecalciferol = 1,580 IU; E = 20 IU; K = 4.05 mg; B12 = 20 μg; biotin = 270 μg; folic acid = 1.62 mg; niacin = 75 mg; pantothenic acid = 12.8 mg; pyridoxine = 4.8 mg; riboflavin = 7.8 mg; thiamin = 3 mg.

However, detailed studies on specific direct effects of VitA deficiency on intestinal immunity in intact broiler chickens are not available. This study was carried out to investigate the role of VitA on the host intestinal immune system and local immune response to an enteric coccidial (*EA*) infection.

MATERIALS AND METHODS

Birds and Diets

Sixty day-old male broiler chicks (Ross 308[3]) were randomly assigned to six cages of an electrically heated battery. All birds were fed milo-soybean meal based diets (Table 1), half with added VitA (8,000 IU all-*trans* retinol per kg of feed) serving as controls (CONT), the other half without added VitA (A-DEF). The diets were formulated to either meet or exceed the recommended National Research Council (1994) nutrient requirements for broilers. On Day 20, birds were weighed individually; light and heavy outliers were discarded and 24 birds (eight per cage) retained on each diet. On Day 21, two birds per cage were selected at random for baseline IEL counts (within diet, counts between cages did not differ; data not reported). On Day 25, serum was collected from two birds per cage (selected on mean BW basis), and those birds were removed to cages in a separate isolation room (still at two birds per cage) for challenge with *EA*. An additional two birds per cage (six per treatment) were retained as unchallenged controls. On Day 28, four birds per treatment were taken at random for collections of spleens, and two birds per treatment discarded (excess). Fecal collections from challenged birds were initiated on Day 31. On Day 35, fecal collection was terminated; serum was obtained from all challenged birds; intestines were taken from challenged and unchallenged birds for IEL.

[3]Allen's Hatchery, Seaford, DE.
[4]FHK, Inc., Tokyo, Japan.
[5]Life Technologies, Grand Island, NY.
[6]Fisher Scientific, Pittsburgh, PA.
[7]HyClone, Pittsburgh, PA.
[8]Robbins Scientific, Sunnydale, CA.

TABLE 2. Monoclonal antibodies used in flow cytometric analysis

Monoclonal antibody[1]	Antigen specificity
CT3	CD3
CTLA4	CD4
CTLA8	CD8
TCR1	$\gamma\delta$ T-cell receptor
TCR2	$\alpha\beta$ T-cell receptor
Anti-IgA	IgA
K55	Pan lymphocytes

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Eimeria Infection and Oocyst Production

At 25 d of age, birds selected for challenge were inoculated orally with 10,000 sporulated oocysts of *EA*. Oocyst production and shedding were assessed as described by Lillehoj and Ruff (1987). Briefly, droppings from each cage (two birds) were collected for 4 d, starting on Day 6 post infection, water was added to each sample, soaked overnight, and the fecal material ground and homogenized in a blade blender. Two 35-mL samples were taken, diluted, and the oocysts were counted microscopically using a McMaster counting chamber.[4] The total number of oocysts was calculated using the formula: total number of oocysts = oocyst count × dilution factor × (fecal sample volume/counting chamber volume)/birds' BW (kg).

Lymphocyte Preparation and Flow Cytometric Analysis

Intestinal IEL were prepared, and changes in their subpopulations determined as described by Lillehoj and Chai (1988). Briefly, small intestinal sections of duodenum and jejunum (two birds per cage, pooled) were excised, cut longitudinally, washed in several changes of ice-cold calcium- and magnesium-free Hank's balanced salt solution[5] (CMF-HBSS), and cut into small pieces. The pieces were treated with 10 m*M* dithiothreitol[6], followed by incubation in 10^{-4} *M* EDTA[6] for 20 min, both in CMF-HBSS supplemented with 5% fetal calf serum[7] (FCS) with continuous swirling at 37 C. The released IEL were passed through a nylon wool[8] column and resuspended in staining buffer (HBSS without phenol red containing 3% FCS and 0.01% sodium azide[6]). Cells were then incubated with mouse anti-chicken monoclonal antibodies (mAb) (Table 2) detecting various lymphocyte subpopulations at 4 C for 40 min, and later with a fluorescein isothiocyanate-

conjugated goat anti-mouse IgG.[9] The cells were then enumerated using a Coulter EPICS XL-MCL flow cytometer[10], and each population expressed as the percentage of total lymphocytes counted.

Serum Antibodies and Interferon-γ ELISA

Individual serum samples (six birds per treatment, each bird serving as its own prechallenge control) taken before and after coccidial challenge were tested for interferon-γ (IFN-γ) and anti-coccidia Ab production using ELISA (Yun et al., 2000a).

Serum IFN-γ was quantified using a direct binding ELISA. Ninety-six well, flat-bottom microtiter plates[11] were coated with 60 μL of sera in 40 μL of 0.1 *M* sodium carbonate buffer, pH 9.6, for 18 h at 4 C, and washed three times with PBS containing 0.05% Tween-20 (PBS-T),[6] pH 7.2. Each well was blocked with 200 μL of PBS containing 2% (vol/vol) BSA[12] for 1 h at room temperature (RT, 20 to 22 C), and washed three times with PBS-T. To each well, 100 μL of mouse anti-chicken IFN-γ mAb were added, incubated for 1 h at RT, and washed three times with PBS-T. Then 100 μL of horseradish peroxidase-conjugated goat anti-mouse IgG (H + L)[12] in PBS-0.1% BSA were added, and the plates incubated for 1 h at RT. The plates were washed three times: 100 μL of 3,3′, 5,5′-tetramethylbenzidine dihydrochloride[12] in 0.05 *M* phosphate-citrate buffer[12], pH 5.0 added for 15 min, the reaction stopped with 50 μL of 2 *M* H_2SO_4,[6] and the optical density (OD) read at 450 nm by an automated microtiter plate reader.[13]

To measure the humoral Ab response to coccidia, microtiter plate wells were coated with 2 μg/well of the recombinant coccidial Ag 3-1E (Lillehoj et al., 2000) in 100 μL of 0.1 *M* carbonate buffer, pH 9.6, for 18 h at 4 C. The plates were washed three times with PBS-T, blocked with 200 μL of PBS-2% BSA for 1 h at RT, and washed as above. Serial dilutions of 100-μL serum samples were added and incubated for 1 h at RT with continuous gentle shaking. The wells were again washed three times with PBS-T, and bound Ab detected with horseradish peroxidase-conjugated rabbit anti-chicken IgG[12] and tetramethylbenzidine dihydrochloride as described above. The plates were also read at 450 nm.

Lymphocyte Proliferation

Spleens were harvested from unchallenged birds in each treatment to measure the mitogenic response of splenic lymphocytes. Lymphocyte preparation and stimulation were performed according to the method outlined by Martin et al. (1994). Freshly removed spleens were homogenized by gently pressing through a stainless-steel mesh, and red blood cells removed by density separation using Histopaque-1077[12]. The lymphocytes were washed by centrifugation in CMF-HBSS, and resuspended to 10^7 cells/mL in Iscove's modified Dulbecco's medium[5] containing 10% FCS. The cells were stimulated by incubating 1 mL of cell suspension with equal volume of 0, 12.5, or 25.0 μg/mL concanavalin A[14] (ConA) in RPMI-1640 Complete media[12] supplemented with 10% FCS. Each sample was incubated in triplicate wells of 24-well cell culture plates at 40 C and 5% CO_2 for 60 h. Following incubation, 100 μL/well of cell suspension were transferred to 96-well U-bottom microtiter plates, 20 μL of dimethylthiazol diphenyltetrazolium bromide[12] (10 mg/mL) solution was added to each well, and the plates were incubated at 37 C for 3 h. The plates were then centrifuged, supernatants discarded, and cells resuspended and lysed by Saponin[12] (10%). Then 175 μL of HCl-isopropanol[6] were added, and OD was determined at 570 nm by an automated microtiter plate reader[13]. A stimulation index for each sample was generated by dividing OD value of ConA-stimulated cells by that of media without ConA.

Statistical Analysis

The experimental design was a 2 × 2 factorial of diet treatments and coccidial challenge. Analysis of variance for the main effects (all parameters) and their interactions (except for splenic lymphocyte proliferation) were tested using the Mixed Model procedures of SAS (SAS Institute, 1996). Means were separated by least significant difference at a $P \leq 0.05$.

RESULTS

Changes in IEL Subpopulations

The differences in the intestinal IEL subpopulations were assessed at 35 d of age (10 d post *EA* challenge). Lymphocytes expressing the surface markers CD3, CD4, CD8, $\alpha\beta$TCR, and $\gamma\delta$TCR, as well as surface IgA are reported as the percentage of total lymphocytes stained with the mAb K55 (Table 2) and presented in Figure 1. Regardless of coccidial challenge status, VitA deficiency resulted in a reduction ($P \leq 0.05$) of all T-cell subtypes tested ($CD4^+$ [helper T cells], $CD8^+$ [cytotoxic T cells], $\alpha\beta TCR^+$, and $\gamma\delta TCR^+$), but not IgA-expressing B cells, which were highest in nonchallenged A-DEF birds. The percentage of $CD3^+$ IEL was reduced ($P \leq 0.05$) due to VitA deficiency. Conversely, there was no effect ($P > 0.05$) associated with the *EA* challenge on the $CD3^+$, $\alpha\beta TCR^+$, and $\gamma\delta TCR^+$ populations. Introduction of *EA* did not affect $CD4^+$ cells in the CONT group, but caused an additional reduction in A-DEF birds, compared to unchallenged A-DEF birds. Further, while $CD8^+$ cells rose ($P \leq 0.05$) in CONT birds in response to the coccidial challenge, an equivalent response did not occur in A-DEF birds.

[9]Southern Biotech, Birmingham, AL.
[10]Coulter Corp., Miami, FL.
[11]Dynex Technologies, Chantilly, VA.
[12]Sigma, St. Louis, MO.
[13]Bio-Rad Clinical Diagnostics, Hercules, CA.
[14]Pharmacia, Piscataway, NJ.

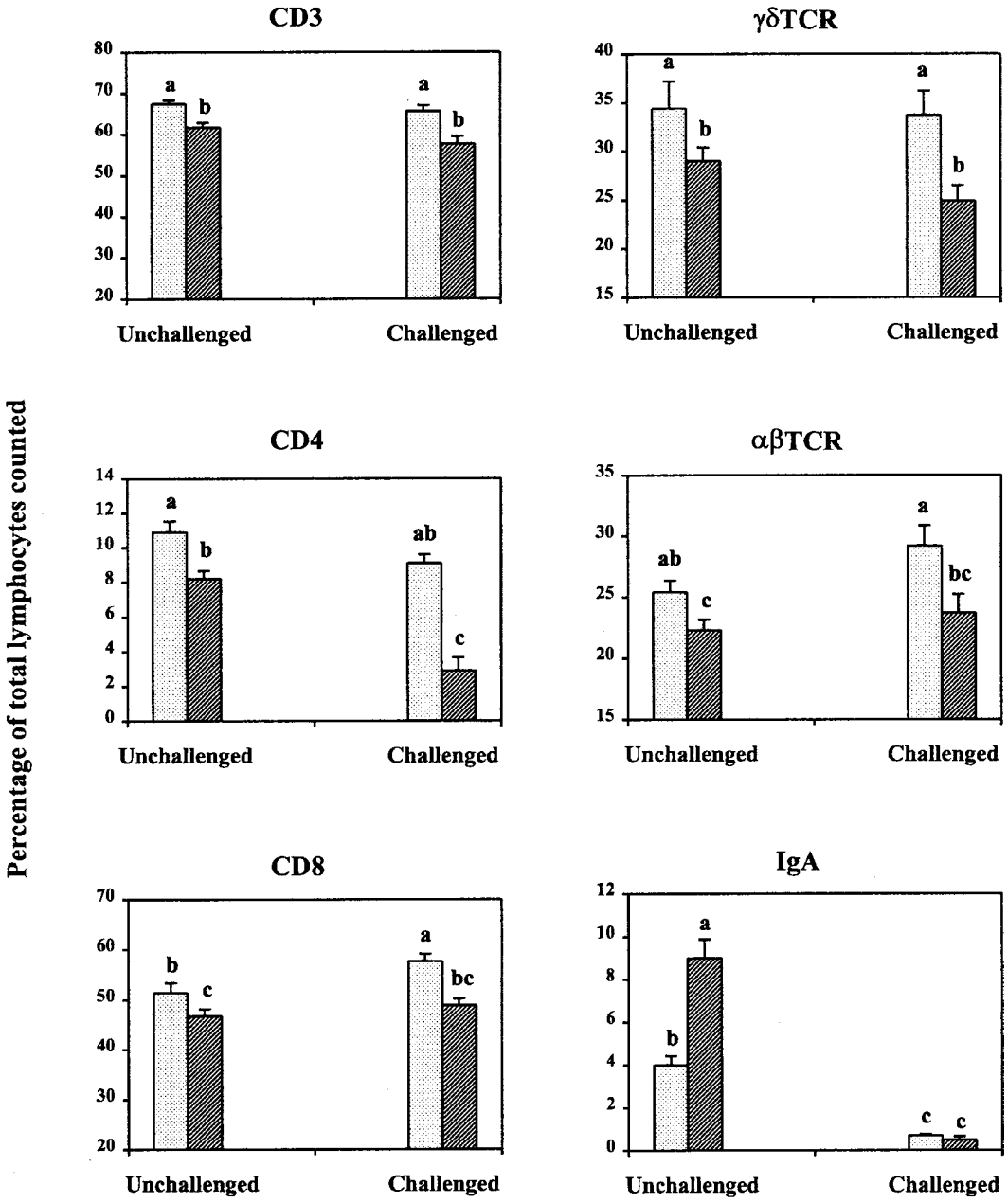

FIGURE 1. Flow cytometric analysis of intraepithelial lymphocytes (IEL) in control (CONT) and vitamin A-deficient (A-DEF) birds without or following *Eimeria acervulina* infection. CONT (stippled bar); A-DEF (striped bar). Challenged birds were given a single oral dose of 10,000 *E. acervulina* oocysts on Day 25 and sampled 10 d later with the unchallenged birds of the same age. The IEL isolated from the upper small intestine (duodenum and jejunum) were analyzed by flow cytometry for the surface antigens CD3, CD4, CD8, $\gamma\alpha$TCR, $\alpha\beta$TCR, and IgA. Data are presented as the percentage of total lymphocytes counted (cells stained with the pan lymphocyte monoclonal antibody K55). Statistical significance ($P \leq 0.05$) is based on ANOVA where the design was a 2 × 2 factorial, with the diet and coccidia challenge as main effects. Each bar represents the mean of six birds. [a-c]Means lacking common letters differ ($P \leq 0.05$).

Assessment of Oocyst Production

Fecal materials from each cage (two birds) were collected for 4 d as described earlier. Due to the significant differences in bird size between the two diet groups, CONT and A-DEF, the total of oocysts collected per cage was divided by the birds' BW per cage on the final day of collection. Data (Means ± SD) are expressed as oocysts/kg BW (Table 3). Control birds shed substantially fewer oocysts ($P = 0.02$) than A-DEF birds (5.4×10^8 vs 9.2×10^8, respectively), despite the higher variation (SD) among the CONT group as compared to the A-DEF birds.

Serum IFN-γ and Anti-Coccidial Antibodies

Serum samples were obtained on day of inoculation and 10 d post inoculation, stored at 20 C, and later tested by ELISA for IFN-γ and for Ab against a recombinant coccidial antigen. Prechallenge samples served as negative controls; therefore, their individual OD values were

TABLE 3. Fecal oocysts[1] shed by birds infected with *Eimeria acervulina*

Diet	Total oocyst number[2] ($\times 10^8$) (Means ± SD)
CONT	5.41 ± 1.6
A-DEF	9.17 ± 0.7

[1]Control (CONT) and vitamin A-deficient (A-DEF) birds were inoculated with 10,000 *Eimeria acervulina* oocysts on Day 25. Fecal samples were collected on Days 6 to 9 post inoculation, and for each sample oocysts were counted in duplicate slides.

[2]Each value represents the mean of oocysts counted from six birds (mean oocysts per kilogram BW). Means differ significantly ($P = 0.02$).

subtracted from those of 10 d post challenge and presented in Figure 2. Serum IFN-γ ELISA readings showed an increased production in CONT birds upon challenge, compared to A-DEF chickens. Conversely, coccidial challenge resulted in a higher Ab response in A-DEF birds than in CONT birds.

Lymphocyte Proliferation

As an indicator of cell-mediated immune competence, mitogenic response of splenic T lymphocytes from 4-wk-old unchallenged birds was measured using the mitogen ConA at two concentrations (12.5 and 25.0 μg/mL). A stimulation index was calculated as the difference in OD values between ConA and non-ConA stimulated cells (Figure 3). Lymphocyte proliferation response to ConA was reduced in A-DEF birds regardless of the ConA concentration used, but the difference was more significant when stimulating with the higher concentration ($P = 0.011$ for 25.0 μg/mL ConA vs. $P = 0.037$ for 12.5 μg/mL ConA).

DISCUSSION

Reduction in the general T-cell population within the intestinal epithelium denotes a weakened local immune system with increased susceptibility to enteric diseases. In the present study, young broiler chicks, A-DEF, exhibited fewer intraepithelial T cells ($CD3^+$) than CONT birds that were fed an adequate level of VitA. This general T-cell effect occurred similarly for all T-cell subpopulations measured. However, T-cell reduction, mainly the $CD4^+$ population, was most affected by the coccidial challenge, suggesting a synergistic interaction between VitA deficiency and coccidial challenge. Reduced $CD4^+$ (T helper) cells would affect other IEL subpopulations involved in local immune responses, through the secretion of cytokines. In this study, VitA deficiency significantly reduced the percentage of $CD8^+$ T cells, which are the main cytotoxic cells in the IEL (Lillehoj and Trout, 1996). The data also showed that no proliferation of $CD8^+$ cells occurred in response to coccidial challenge in A-DEF birds, a further indication of impaired local immune responses due to VitA deficiency.

Lessard et al. (1997) examined the effects of different VitA levels on the immune responsiveness of broiler chickens to Newcastle disease virus (NDV) post-immunization and demonstrated a significantly lower percentage of splenic $CD4^+$ T lymphocytes in chickens fed a low-VitA diet (400 IU/kg) as compared to those on higher VitA levels. This resulted in significantly lower CD4:CD8 T-cell ratios in those birds on the low-VitA diet. Our data similarly show a VitA deficiency-dependent lowering of the CD4:CD8 ratio in lymphocytes in intestinal epithelium. Failure to recruit $CD8^+$ IEL in A-DEF birds suggests a lack of recruitment signals in the form of cytokines. In this study, the production of IFN-γ, normally produced by a subset of $CD4^+$ T- cells (Yun and Lillehoj, 2000), was depressed in A-DEF in comparison to CONT birds. This becomes crucial in presence of enteric pathogens such as *Eimeria* invading the intestinal epithelial lining where $CD8^+$ T lymphocytes have been shown to play an important role in immune responses to these parasites (Lillehoj and Bacon, 1991; Yun and Lillehoj, 2000).

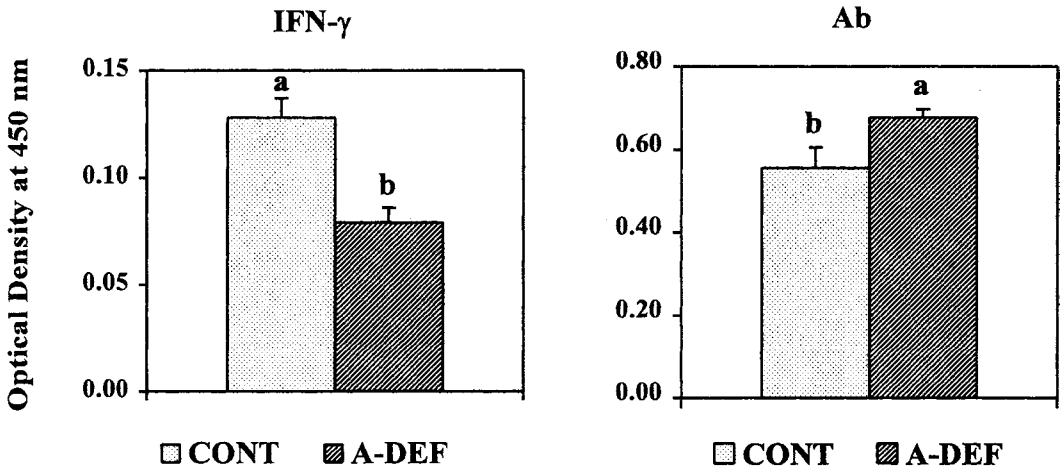

FIGURE 2. Serum interferon-γ (IFN-γ) and anti-coccidial antibody (Ab) responses following *Eimeria acervulina* challenge (CONT = control; A-DEF = vitamin A-deficient). Each bar represents an average ELISA reading (OD = optical density) of immune sera (six samples per treatment group, tested in duplicate wells) obtained 10 d post challenge. Each OD reading (at 450 nm) was calculated by subtracting the background reading (from prechallenge sera) from the reading of immune sera (infected birds). [a,b]Means lacking common letters differ ($P \leq 0.05$).

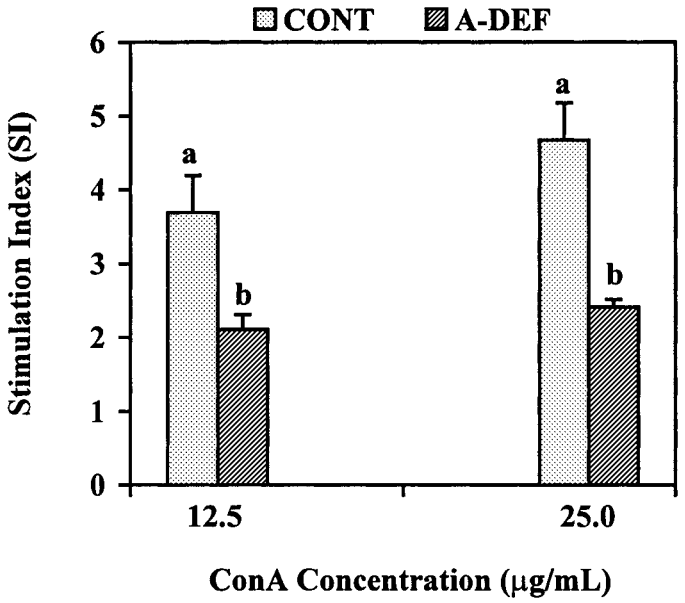

FIGURE 3. Response of splenic lymphocytes to concanavalin A (ConA) (CONT = control; A-DEF = vitamin A-deficient). Stimulation Index (SI) = optical density (OD) (at 570 nm) of cells with ConA/OD of cells without ConA. Each bar represents an average of four samples per group. Means lacking common letters differ ($P \leq 0.05$).

Protective roles intestinal IEL play can be estimated by measuring the number of oocyst shed. Control birds shed fewer *EA* oocysts than the A-DEF group, a clear indication of higher susceptibility of broiler chickens to this parasite due to VitA deficiency. Two factors may explain this result: first, the reduction in $CD4^+$ IEL and the subsequent effect on $CD8^+$ T cells diminish the extent of immune response against the invading parasite. Second, since VitA functions to maintain epithelial tissues (Uni et al., 1998), a lack of this vitamin, by reducing the integrity of the epithelial barrier, can lead to easier penetration of *Eimeria* into the intestinal epithelium, its proliferation site. This is in accord with Singh and Donovan (1973), who found that oocyst production and dietary VitA levels are inversely related. They attributed that to lower resistance of the intestinal epithelium in A-DEF birds, resulting in the eimerian sporozoites readily gaining access to the epithelium and then to the submucosa of the intestinal walls for their development. Vitamin A deficiency lowers resistance of epithelium and results in keratinization and degeneration of mucus-secreting epithelial cells (Moore, 1957). It also interferes with the synthesis of mucopolysaccharides covering the intestinal epithelial layer (Wolf and Varandani, 1960). Uni et al. (2000) showed that lack of VitA reduced the percentage of goblet cells in the intestinal villi of broiler chickens. Similarly, De Luca and Wolf (1972) and Reifen et al. (1998) showed a marked decrease in mucus-secreting goblet cells in A-DEF rats.

Cytokines have been used as measures of cellular immunity to coccidial infection (Lillehoj and Trout, 1996; Yun and Lillehoj, 2000); in particular, IFN-γ has been used as a common marker of T-cell responses to *Eimeria* (Martin et al., 1994; Yun et al., 2000b). In the present study, assay of serum IFN-γ showed this cytokine to be higher in CONT birds upon challenge compared to A-DEF chickens. This is consistent with the theory that a reduction in the percentage of $CD4^+$ IEL or T-helper cells can alter the profile of cytokines secreted at the infection site. Vitamin A deficiency has been shown in some cases to influence the balance between T-helper type 1 (Th1) and T-helper type 2 (Th2) immune responses (Mossmann and Coffman, 1989). In mice, Th1-type responses are mainly characterized by enhancement of certain cytotoxic mechanisms and inflammatory reactions partly due to increased IFN-γ production. On the other hand, during Th2-type responses, the production of other cytokines such as interleukin (IL)-4 helps in the activation and proliferation of B cells and triggering a strong Ab response. *Trichinella spiralis* infection in mice was found to stimulate a strong Th2-like response with high levels of parasite-specific IgG and a cytokine profile characterized by IL-4, IL-5, and IL-10 production. In contrast, A-DEF mice infected with *T. spiralis* produced Th1-like responses with low production of parasite-specific IgG and cytokine profile characterized by IFN-γ and IL-12 production (Carman et al., 1992; Cantorna et al., 1994; 1995). Although Th1-type and Th2-type subsets have not been described in chickens, Lessard et al. (1997) suggested that A-DEF chickens developed a Th2-type immune response, whereas those birds fed a high-VitA diet showed a Th1-type response following NDV vaccination. Our results appear in agreement with the latter findings in terms of IFN-γ production.

Lessard and co-workers (1997) also reported a higher antibody response to NDV vaccine in birds kept on low VitA than in birds fed adequate or high VitA. Although the challenge in this study differs, we also observed a higher Ab response in A-DEF birds than in CONT chickens following the coccidial infection. This observation further suggests an induction of systemic Th2-type immune response in A-DEF chickens. However, an equivalent response did not seem to occur locally, since a very small percentage of IEL expressed surface IgA in both CONT and A-DEF birds. In contrast, Davis and Sell (1989) and Sklan et al. (1994) reported lower Ab responses to NDV vaccination and β-casein injection, respectively, in A-DEF than in VitA-sufficient chickens.

The A-DEF birds showed a depressed mitogenic response to ConA regardless of the ConA concentration used. In contrast to our results, Lessard et al. (1997) reported higher overall responses to ConA by splenic lymphocytes isolated from chickens raised on low-VitA diet than responses in birds on adequate or high-VitA diets. However, their tests followed a NDV vaccination. Other researchers showed reduced lymphocyte proliferation in response to different mitogens in VitA-deficient chickens (Friedman and Sklan, 1989a; Sklan et al., 1994). T lymphocytes are the major regulatory cells of the adaptive immune system, and their activation has been reported to require retinol, a form of VitA (Garbe et al., 1992). Therefore VitA deficiency can affect the T-cell populations being activated and, consequently, the type and degree of immune response developed against a disease challenge.

In conclusion, VitA deficiency was shown to impair the local immune defenses within the GALT of broiler chickens. This effect was best characterized by the changes in IEL subpopulations, mainly $CD4^+$ or T-helper cells, as they are responsible for orchestrating many other cellular immune responses through cytokine secretions and interaction with other immune cells. The results of this study showed that alteration in the IEL subpopulations and reduced local cell-mediated immunity caused by lack of VitA lowered the ability of broilers to resist *EA* infection. NRC (1994) has defined VitA requirements for optimal growth and performance of broiler chickens; however, the optimal levels for best immune system development and function, both locally and systemically, remain to be determined.

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